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Expression and Its Role in Outgrowth of Facial Prominences and Limb Buds

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Embryonic facial development in chick embryos involves a sequential activation of genes that control differential growth and patterning of the beak. In the present study we isolate one such gene, the transcription factor, AP-2, that is known to be expressed in the face of mouse embryos. The protein sequence of chick AP-2α is 94% homologous to human and mouse AP-2. Wholemount *in situ* hybridization with a probe for chick AP-2 identifies expression from primitive streak stages up to stage 28. The most striking expression patterns in the head are during neural crest cell migration when AP-2 transcripts follow closely the tracts previously mapped for neural crest cells. Later, expression in the facial mesenchyme is strongest in the frontonasal mass and lateral nasal prominences and is downregulated in the maxillary and mandibular prominences. Once limb buds are visible, high expression is seen in the distal mesenchyme but not in the apical ectodermal ridge. The expression patterns of AP-2 in stage 20 embryos suggested that the gene may be important in "budding out" of facial prominences and limb buds. We implanted beads soaked in retinoic acid in the right nasal pit of stage 20 embryos resulting in a specific inhibition of outgrowth of the frontonasal mass and lateral nasal prominences. AP-2 expression was completely down-regulated in the lateral nasal within 8 hr of bead application. In addition, the normal up-regulation of AP-2 in the frontonasal mass did not occur following retinoic-acid treatment. There was an increase in programmed cell death around the right nasal pit that accompanied the down-regulation of AP-2. Prominences whose morphogenesis were not affected by retinoic acid did not have altered expression patterns. We removed the apical ectodermal ridge in stage 20 limb buds and found that AP-2 expression was partially downregulated 4 hr following ridge removal and completely downregulated 8 hr following stripping. Application of an FGF-4 soaked bead to the apex of the limb bud maintained AP-2 expression. Thus AP-2 is involved in outgrowth and could be regulated by factors such as FGFs that are present in the ectoderm of both the face and limb.

INTRODUCTION

One of the great challenges in developmental biology is to understand the control of patterning in the embryo. It is likely that some conserved regulatory pathways are deployed at several different stages of development and at several different sites. One example of this may be in craniofacial and limb development where several molecules have been identified that are expressed in both locations. One such molecule is the transcription factor activating protein-2 (AP-2).

AP-2 can bind to the promoter of approximately 100 genes via a conserved palindromic sequence (5′-GCCN$_3$-GGC-3′; Mitchell *et al.*, 1987; Mitchell and Tjian, 1989; Williams and Tjian, 1991). Isolation of the mouse AP-2 genes and analysis of highly localized expression patterns in embryos raised the possibility that AP-2 is important for certain aspects of embryogenesis (Mitchell *et al.*, 1991; Chazaud *et al.*, 1996). In particular, expression of AP-2 transcripts in the cranial neural crest, facial prominences, and limb buds suggested that AP-2 could be an important regulatory gene for face and limb develop-

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ment. Indeed, knockout mice lacking functional AP-2 protein have severe dismorphology of the face and phocomelic forelimbs (Schorle *et al.*, 1996; Zhang *et al.*, 1996). In order to further study the function of AP-2 in face and limb development we turned to the chick model system which offers several assays for studying gene function in manipulated embryos.

The chick face is formed during four critical periods of embryonic development: (1) cranial neural crest cell formation [3 somites, stage 8− (Hamburger and Hamilton, 1951)] neural crest cell movement (3–17 somites, stages 8− to 12), (3) proliferation of facial mesenchyme (stages 15 to 24), and (4) fusion and differentiation of the facial prominences (swellings of mesenchyme covered in epithelium which surround the primitive oral cavity; stages 28 to 30). The cranial neural crest moves away from the posterior forebrain, midbrain, and anterior hindbrain in an antero-posterior wave starting at 3 somites (Lumsden *et al.*, 1991; Couly and Le Douarin, 1993). The cells move from the dorsal mesencephalon ventral into the upper face, skirting the eyes (Johnston, 1966; Lumsden *et al.*, 1991). Cells from the posterior mesencephalon and anterior rhombencephalon move into the first branchial arch, ending by stage 15 (Lumsden *et al.*, 1991; Kontges and Lumsden, 1996).

Few genes are known to be expressed in cranial regions of the migrating neural crest, anterior to rhombomere 2 (Hoxa-2 is the most anteriorly expressed homeobox gene; Prince and Lumsden, 1994) and these are members of the muscle-specific homeobox family (Msx-1; Lyons *et al.*, 1992), RXR-γ (retinoid X receptor-γ; Rowe and Brickell, 1995), and *Slug*, a zinc finger transcription factor (Nieto *et al.*, 1994). Therefore it is important to study AP-2 which is expressed in murine forebrain- and midbrain-derived neural crest cells and subsequently in facial prominences (Mitchell *et al.*, 1991 Chazaud *et al.*, 1996)

The developing facial prominences and limb buds both arise from buds of mesenchyme encased in epithelium. The limb bud mesoderm is derived from lateral plate mesoderm and buds out from the body wall starting at stage 17 under the influence of ectodermal signals, probably members of the fibroblast growth factor family (Cohn *et al.*, 1995; Crossley *et al.*, 1996; Vogel *et al.*, 1996). Later, once the limb bud is established, the apical ectodermal ridge (AER) is responsible for maintaining outgrowth of the limb (Saunders, 1948; Summerbell, 1974). Facial prominences also require ectoderm for outgrowth (Wedden, 1987; Saber *et al.*, 1989; Richman and Tickle, 1989), although there are no obvious ridges similar to the AER in facial ectoderm (Wedden *et al.*, 1988). Facial ectoderm can support outgrowth and digit formation in limb bud mesenchyme (Richman and Tickle, 1992), suggesting that some signals passing between epithelium and mesenchyme are similar in these two parts of the embryo.

Our present study investigates the relationship between outgrowth and maintenance of expression of AP-2 in the facial prominences and wing bud. Chick embryos exposed to retinoic acid have truncations of the frontonasal mass and develop clefts of the beak (Tamarin *et al.*, 1984; Wedden and Tickle, 1986; Richman and Leon Delgado, 1995). There is a lag of about 24 hr before the first changes in shape of the upper beak prominences can be detected thus early changes in gene expression can be studied in this time window. The molecular causes of the failure in outgrowth of the frontonasal mass are not clear but one possibility is that AP-2 expression is altered which in turn modifies expression of factors important for facial growth. The present study examines the expression of AP-2 following treatment of the chick face with retinoic acid and links the process of outgrowth of the facial prominences with decrease in AP-2 expression and a concomitant increase in programmed cell death. In a related experiment, we remove the apical ectodermal ridge and study the expression of AP-2 at defined intervals following ridge removal. Our results on both the face and limb demonstrate that AP-2 expression is downregulated if outgrowth is inhibited.

MATERIALS AND METHODS

Animals

Fertile white leghorn chicken eggs were purchased from Coastline Chicks (Abbotsford, B.C.) and incubated at 38°C until the desired stage was reached. Embryos were staged according to the criteria of Hamburger and Hamilton (1951).

Cloning of AP-2

A stage 24 chick head cDNA library was synthesized in Lambda Zap-II (Stratagene) and screened with a 240-bp mouse cDNA (Mitchell *et al.*, 1991). The primary screen resulted in 3 positive clones, all with a 1858-bp insert. One of the clones, 1.1, was grown up and sequenced using the Sanger method. The sequencing was partly carried out by the Dept. of Molecular Biology, Simon Fraser University. Sequence analysis was performed with the BLAST program. A subclone of the gene removing 600 bp of 3′ sequence (cut at the StyI site) was generated for use in expression studies (Fig. 2A).

Southern and Northern Blotting

Genomic DNA was isolated from chick embryos and digested with *Eco*RI, *Hin*dIII, *Pst*I, or *Bam*HI. The gel was transferred to supported nitrocellulose membrane (MSI) and probed with [^{32}P]-dCTP-labeled, 1.3-kb StyI fragment of the chick cDNA. Following hybridization, the blot was washed to a maximum stringency of 0.1× SSC, 0.5% SDS, 55°C for 10 min, twice, and exposed to film overnight.

Stage 24 frontonasal mass facial prominences were dissected including all tissue between the nasal pits (Richman and Crosby, 1990). Total RNA was isolated according to the method of Chomc-

zinski and Saachi (1987) and 20 μg was separated on a 1% agarose/Mops-formaldehyde gel. The gel was blotted onto supported nitrocellulose (MSI). To generate probes, inserts from AP-2 were gel purified and labeled with [^{32}P]dCTP using a random prime kit (Pharmacia). The blots were hybridized overnight at 42°C in 50% formamide and washed to a maximum stringency of 0.1× SSC, 65°C, 15 min, prior to exposure to X-ray film for 2 days.

Embryo Manipulations

Bead implantation in the nasal pit. AG1X-2 beads, 100 μm in diameter (formate form, Bio-Rad) were soaked in 5 mg/ml of all-*trans*-retinoic acid dissolved in dimethylsulfoxide (DMSO) and rinsed in medium with 10% fetal calf serum (Gibco) as described in Richman and Leon Delgado (1995). Beads were implanted into the right nasal pit of stage 20 embryos and they were incubated 4, 8, 16, 24, or 48 hr prior to fixation. Control embryos were treated with DMSO-soaked beads.

AER removal and FGF-4 bead implantation. Stage 20 embryos were used for these experiments. The apical ectodermal ridge (AER) was stained with neutral red, carefully elevated with a fine tungsten needle, and then excised. In some cases a bead soaked in 1 mg/ml FGF-4 (Genetics Institute) was stapled to the apex of the stripped wing using platinum wire. Embryos were incubated a further 4, 6, 8, 12, and 24 hr and then fixed for wholemount *in situ* hybridization.

Wholemount Immunocytochemistry with the AP-2 Antibody

Procedures as described in Zhang *et al.* (1996) and Mark *et al.* (1993) were used. Briefly, the monoclonal antibody 3B5, which recognizes an AP-2α-specific epitope in the mouse (Zhang *et al.*, 1996), was also utilized for the chicken studies. Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline and then incubated in 0.1% hydrogen peroxide, 1% Triton X-100, 10% fetal calf serum in PBS overnight at 4°C to inactivate endogenous peroxidases. Embryos were incubated in the supernatant for 3 days at 4°C and presence of antibody was detected with a peroxidase-conjugated anti-mouse IgG.

Wholemount in Situ Hybridization

The methods of Izpisúa-Belmonte *et al.* (1993), Hemmati-Brivanlou and Harland (1990), and Henrique and Ish-Horowitz (personal communication, C. D. Stern) were used for these experiments. Embryos were fixed in 4% formaldehyde in PBS, 2 m*M* EGTA overnight, processed into 100% methanol, and stored at −20°C until use. Embryos stage 10 and younger were treated for up to 10 min in 10 μg/ml of proteinase K. Embryos stage 12–18 were treated for 25 min in 10 μg/ml proteinase K. Stages 20, 24, and 28 embryos were treated for 15, 20, and 25 min, respectively, with 20 μg/ml proteinase K. Hybridization was carried out with 0.5 μg/ml digoxygenin-labeled probe, 50% formamide, 1.3× SSC (pH 4.5), 50 μg/ml yeast RNA, 0.2% Tween 20, 0.5% Chaps, 100 μg/ml heparin at 70°C for 18 hr. High stringency washing conditions were used to a maximum of 0.65× SSC/0.25% Chaps/25% formamide at 70°C. Following detection with the DIG antibody (Boehringer Mannheim) embryos were photographed with a Leica dissection microscope using Fuji color print film and dark field or bright field illumination. Embryos were refixed in 10% buffered formalin and selected specimens were embedded in wax and sectioned at 10 to 12 μm to visualize tissue localization of the probe. In some instances sections were counter stained with 0.1% Malachite green. Sections were photographed with a Zeiss compound microscope.

In Situ Hybridization on Sections

Embryos were fixed in 4% paraformaldehyde/PBS, processed through ethanols, and embedded in wax. The AP-2 probe used was the *Sty*I fragment described above. Another probe generated from a 231-bp fragment (nucleotides 939–1170) located in the DNA binding/dimerization domain, did not show specific hybridization patterns. The methods used for preparing [^{35}S-UTP]-labeled probe and hybridization were identical to those described in Rowe *et al.* (1991) and Richman and Delgado (1995).

Detection of Programmed Cell Death

We used the TUNEL method (terminal deoxynucleotide transferase [TdT]-mediated dUTP-digoxygenin nick-end-labeling) to detect programmed cell death in sections and in wholemounts. For TUNEL on sections, the methods of Wride *et al.* (1994) were used. Embryos were fixed in 4% paraformaldehyde/PBS, embedded in wax, and sections from normal and retinoic acid treated embryos were placed on slides together. Following dewaxing and rehydrating slides were incubated in 2× SSC (0.3 *M* NaCl, 30 m*M* sodium citrate, pH 7) at 60°C for 15 min, treated with 20 μg/ml proteinase K at room temperature for 15 min, refixed in 4% paraformaldehyde/PBS for 20 min, rinsed, and then incubated in the terminal deoxytransferase buffer (140 m*M* sodium cacodylate, 1 m*M* cobalt chloride, 30 m*M* Tris–HCl, pH 7.2) for 5 min. Terminal deoxytransferase (0.3 U/μl and digoxygenin-labeled dUTP (10 μ*M*, Boehringer Mannheim) were added to the buffer and this solution was added to the sections. A coverslip was placed overtop and the slides were incubated at 37°C for 1.5 hr. Negative control sections did not have the enzyme and positive control sections were pretreated with DNAse I (0.06 U/μl). The DNAse I buffer consisted of 40 m*M* Tris–HCl, pH 7.5, 6 m*M* magnesium chloride, and 0.1 m*M* dithiothreitol. Detection of the label was achieved by incubating the slides with an anti-digoxygenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) at 4°C overnight followed by incubation in substrate for alkaline phosphatase (BCIP and NBT). Slides were coverslipped and photographed under brightfield illumination.

Wholemount TUNEL methods were based on those of Conlon *et al.* (1995). Embryos were fixed in 4% paraformaldehyde/PBS overnight, dehydrated in a series of methanol/PBS solutions, and stored at −20°C in 100% methanol. Embryos were rehydrated to PTW (PBS with 0.1% Tween 20), treated with proteinase K (20 μg/ml) 20 min at room temperature, refixed in 0.2% gluteraldehyde and 4% paraformaldehyde in PTW, incubated in 0.1% sodium borohydrate for 20 min at room temperature, washed in TdT buffer (as described for sections) for 5 min, and incubated in TdT buffer containing 20 μ*M* dig-dUTP and 0.3 U/μl TdT for 2 hr at 37°C. Embryos were incubated in TBST (140 m*M* NaCl, 2.7 m*M* KCl, 25 m*M* Tris–HCl, 0.1% Tween 20) at 70°C for 20 min, to inactivate endogenous alkaline phosphatases, and then

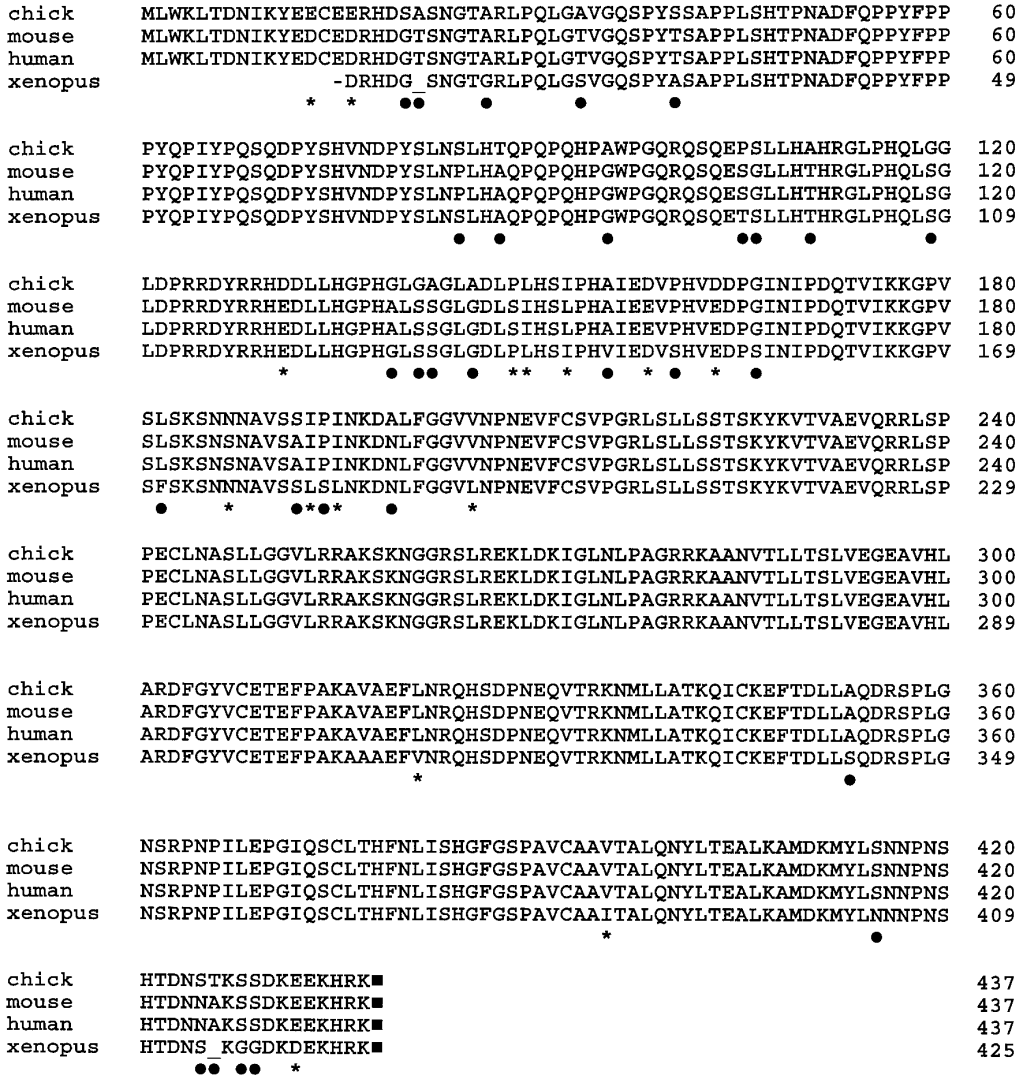


FIG. 1. Comparison of the amino acid sequence chick AP-2 to those of human, mouse, and Xenopus. The Xenopus AP-2 is shown from the sixth amino acid. Dashes were introduced to preserve sequence overlap. Amino acid sequence variation is indicated by ●. Similar amino acids are indicated by asterisks (*). Translation stop codons are represented by boxes (■).

antibody detection as described in the wholemount *in situs* was carried out.

RESULTS

Cloning and the Primary Structure of Chick AP-2α

A cDNA clone for chick AP-2α was isolated containing a predicted open reading frame of 437 amino acids that is 94% conserved relative to human and mouse AP-2α (Williamson *et al.*, 1996) and 92% homologous to xenopus proteins, respectively (Winning *et al.*, 1991; Fig. 1). We therefore concluded that we had isolated the chick AP-2α cDNA. The region of highest homology (99%) is that spanning the DNA binding and dimerization domains (aa 200 to 437). In contrast, there is more variation in the region spanning amino acids 20 to 200 (87% conserved), a region that harbors the transactivation domain (Williams and Tjian, 1991); however, most of the proline and glutamine residues in this area are located at identical positions. The predicted protein has a molecular weight of 48 kDa, very similar to the size determined for human AP-2α (Williams *et al.*, 1988).

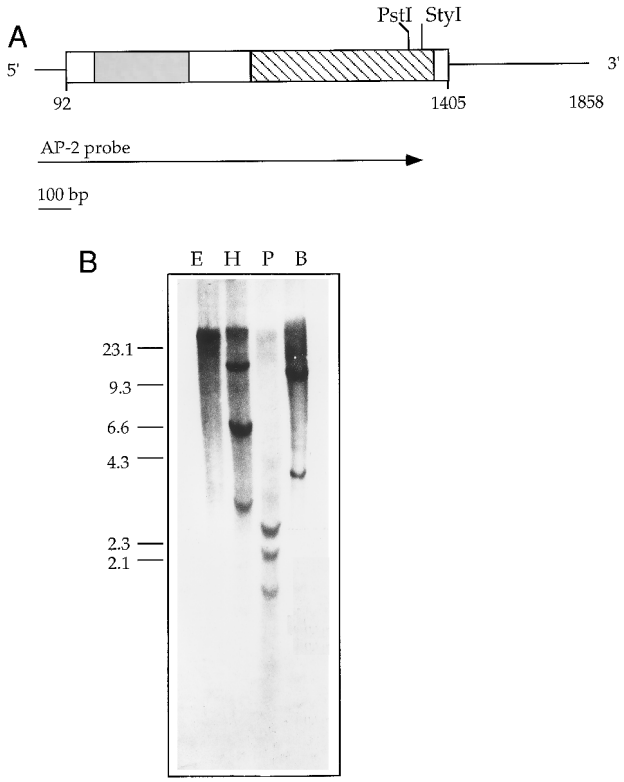


FIG. 2. (A) Schematic representation of AP-2 cDNA clone. Box represents AP-2 coding region: shaded area, the transactivation domain; hatched area, the DNA binding and dimerization domains. *Sty*I site was used for the hybridization probe. The probe is represented by an arrow. (B) Southern analysis of chick genome. Restriction enzymes used are indicated on top of each lane: E (*Eco*RI), H (*Hin*dIII), P (*Pst*I), and B (*Bam*HI).

The nucleotide sequence of the chick AP-2α coding region is 84, 83, and 78% identical to human, mouse and Xenopus AP-2α DNA, respectively. Our chicken clone is not similar to the variants of mouse AP-2α that have been identified (Meier *et al.*, 1995). The cDNA clone contains 91 base pairs of 5′ untranslated sequence and there are two possible start sites for protein translation. The 3′ untranslated sequence consists of 453 base pairs (GenBank Accession No. U72992).

The expression of AP-2 was examined by Northern blot analysis of total RNA from stage 24 chicks using the 1.3-kb *Sty*I fragment (Fig. 2A) as the probe. A major band of 4.3 kb was detected (data not shown), a larger transcript than those reported for human, mouse, and Xenopus (3.3–3.6, 1.8 to 3, and 1.8 to 2.6, respectively; Williams *et al.*, 1988; Moser *et al.*, 1993; Winning *et al.*, 1991). The 1.9-kb cDNA clone we isolated is probably not full-length. There are no hairpin or direct repeats in the 5′ untranslated region; these, if any exist, are likely to be further upstream.

Southern analysis of genomic DNA using the same probe as in the Northern blot and moderately stringent washing conditions, identified one large fragment of about 40 kb in *Eco*RI-digested DNA (Fig. 2B), indicating a single copy of the chick AP-2 gene in the genome. However, some fragments with faint intensity were also observed in this lane, suggesting more AP-2 genes with less sequence homology may exist in the chick genome. We expect that our probe will recognize other AP-2 genes (Moser *et al.*, 1995; Williamson *et al.*, 1996) in addition to AP-2α, if these exist in the chicken.

Expression of AP-2 in Early Gastrulating Embryos

A minimum of 3 embryos were examined for each developmental stage. In stage 5 embryos AP-2 transcripts were localized to the anterior and lateral epiblast but not in the hypoblast, presumptive neural plate, primitive streak, Hensen's node, or the forming mesoderm (Figs. 3B and 3C). In 3–4 somite embryos (stage 8−) expression was restricted to the ectoderm lateral and anterior to the neural plate in the subcephalic pocket (Figs. 3E–3G). AP-2 protein was expressed in the same pattern as the RNA transcripts (Figs. 3A and 3D).

AP-2 Is Expressed in Presumptive Premigratory and Migratory Neural Crest Cells

In 5–8 somite embryos (stage 8+) there was expression of AP-2 at the dorsal edges of the neural folds in the cranial region down to the 4th or 5th somite and in the lateral ectoderm of the tail region (data not shown). It was not possible to detect expression lateral to the neural folds and in the mesoderm until later stages. In stage 10 embryos (10 somites, Fig. 4A), AP-2 transcripts were seen in a diffuse group of cells flanking the neural tube, extending from the posterior diencephalon down to and including the second rhombomere (r2). Sections of these embryos revealed expression in the mesenchyme as well as surface ectoderm and in the dorsal midline of the neural tube (not shown). In the rhombencephalon there were two regions with lower expression of AP-2 and these coincided with r3 and r5 (Fig. 4A). There was expression caudal to the hindbrain in the dorsal midline of the neural tube tapering off by the 6th somite. In stage 12 embryos the pattern of AP-2 transcripts was more defined (Figs. 4B and 4D–4F). AP-2 expressing cells were lateral to, but did not lie directly over the dorsal midline of the mesencephalon. There were AP-2 expressing cells along the midline of the hindbrain extending from region of r2 to r8 (Fig. 4B). In addition, there were two streams of AP-2 expressing cells emanating from r4 and r6 and there were no AP-2 expressing cells adjacent to r3 and r5 (Fig. 4B). Sections of the embryo in Fig. 4B reveal that expres-

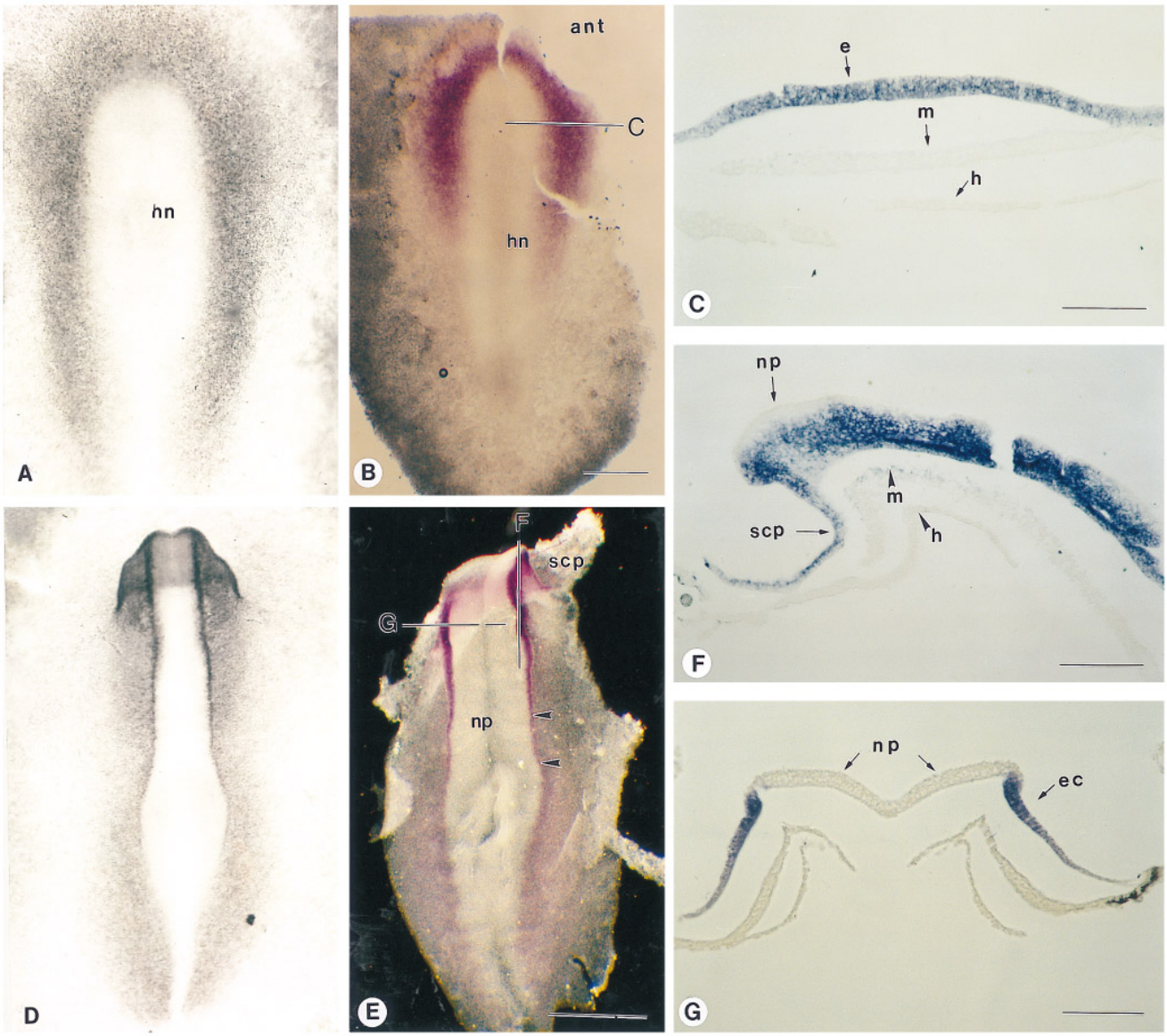


FIG. 3. Expression of AP-2 in gastrulating and early neurula stage embryos by *in situ* hybridization with AP-2 anti-sense probe or by an antibody to human AP-2. Approximate planes for sections in C, F, and G are shown in A and D with horizontal or vertical lines. Scale bar, 500 μm in A, B, D, E; 100 μm in C, F, and G. Key: (e) ectoderm, (h) hypoblast, (hn) hensen's node, (m) mesoderm, (np) neural plate, (scp) subcephalic pocket. (A) Stage 4 embryo showing AP-2 protein expression in a horseshoe shape surrounding the presumptive neural plate. (B) Expression of RNA in a stage 5 embryo anterior and lateral to the presumptive neural plate, not extended as far caudal as in A. (C) Transverse section through the embryo in A. High expression in the epiblast, no expression in the mesoderm or hypoblast. (D) Stage 8 embryo shows AP-2 protein is expressed lateral to the neural plate. (E) In stage 8 embryos (3 somites) there is high expression of AP-2 transcripts at the junction of the ectoderm and neural plate (arrowheads) and no expression in the greater area of the neural plate similar to the protein pattern in D. (F) Parasagittal section of stage 8 embryo showing expression in the neural ectoderm and in the subcephalic pocket. (G) Transverse section shows expression in the surface ectoderm lateral to the neural plate.

sion of AP-2 was present in the mesenchyme surrounding the eye primordia, in the ectoderm that will form the lens and in the presumptive branchial arch region (Figs. 4D and 4E). In addition there was strong expression in the lateral surface ectoderm and no expression in the neural epithelium of the brain (Figs. 4D and 4E). The

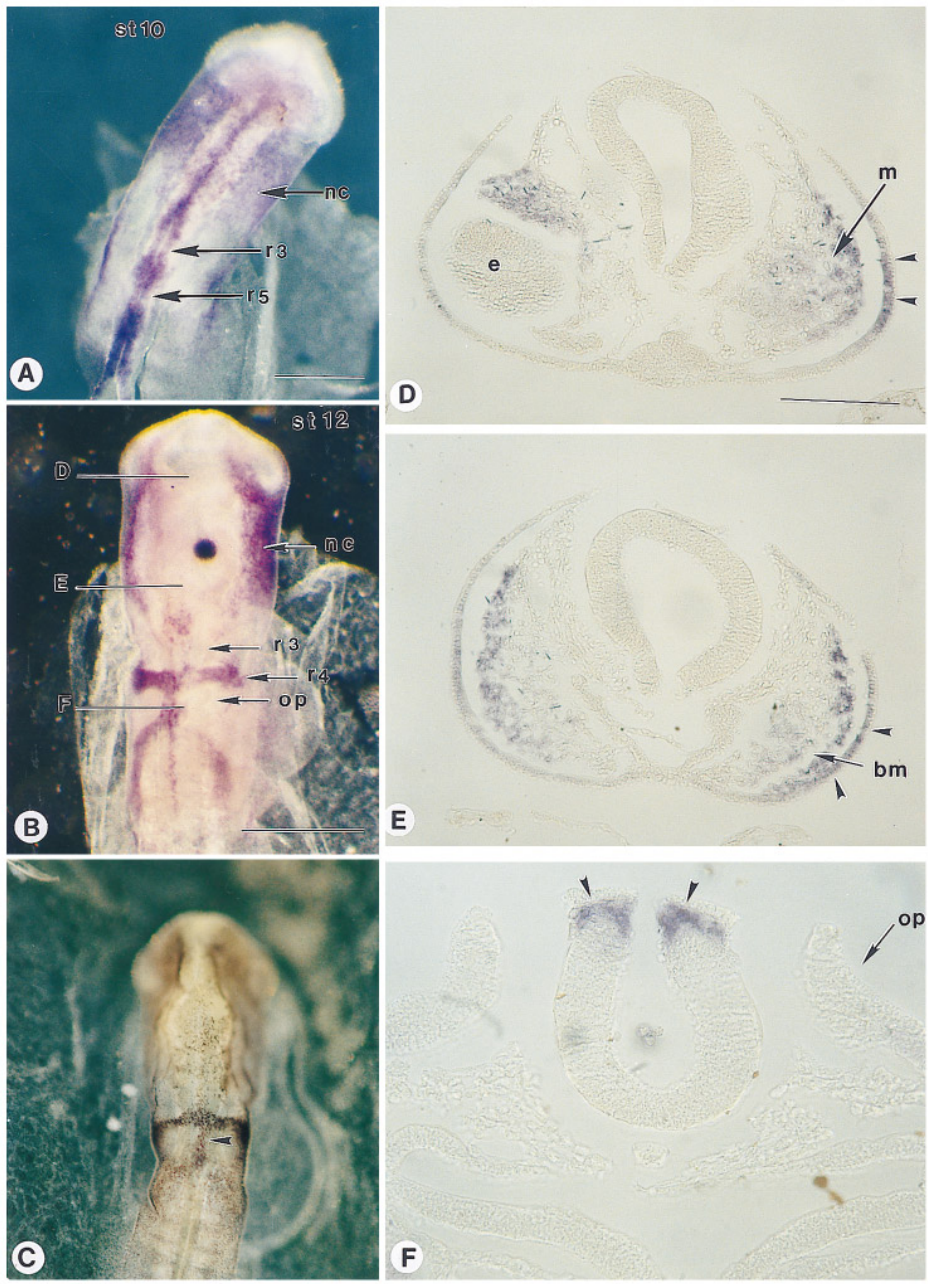


FIG. 4. AP-2 expression in presumptive premigratory and migratory neural crest cells. Approximate planes for sections in D, E, and F are shown in B with horizontal lines. Scale bar, 500 μm in A, B, and C; 100 μm in D, E, and F. Key: (bm) branchial arch mesenchyme; (e) eye, (m) mesenchyme, (nc) presumptive neural crest cells, (op) otic placode, (r3, r4, or r5) rhombomere 3, 4, or 5, (st) stage. (A) Stage 10 embryo. Expression at the dorsal midline of the neural tube and lateral to the neural tube. (B) Stage 12 embryo. Expression flanking the mesencephalon, the dorsal midline of the hindbrain, and streams of AP-2 expressing cells adjacent to r4 and r6. (C) Stage 12 embryo. AP-2 antibody staining shows the protein is expressed in the same pattern as the RNA including along the dorsal midline of the neural tube in the hindbrain (arrowhead). (D) Stage 12, transverse section through the embryo in B. Expression in mesenchyme and surface ectoderm that will form the lens (arrowheads). (E) Stage 12, transverse section through the presumptive branchial arch region. Expression in mesenchyme and ectoderm (arrowheads). (F) Stage 12, transverse section through the otic placodes at the level of r5. Expression at the dorsal edges of neural folds (arrowheads).

section through the otic placode (Fig. 4F) showed strong AP-2 signal at the dorsal edges of the neural tube, but no expression in the mesenchyme or ectoderm lateral to the neural tube. In the presumptive tail, AP-2 expression was found at the lateral edges of the ectoderm covering the unsegmented mesoderm (data not shown). AP-2 antibody staining in a stage 12 embryo overlapped the pattern of RNA transcripts (Fig. 4C).

AP-2 Is Expressed in the Craniofacial Region during Important Stages of Face Development

Once the embryo has turned (stage 13), expression was visible in 2 streams of cells skirting the otic vesicle and in the proximal mesenchyme of the first branchial arch (Fig. 5A and data not shown). Between stages 17 and 20 there was also expression above background levels in the ventral mesenchyme of the upper face between the nasal pits, in the 1st, 2nd, 3rd, and 4th branchial arches and in the trigeminal ganglion (Figs. 5B and 6A and data not shown). AP-2 transcripts were present in the surface ectoderm of the head, but not in the nasal placodes (Fig. 5B and data not shown). Sections of the branchial arches revealed higher levels of AP-2 transcripts in the peripheral mesenchyme than in the mesodermally derived core (not shown). In more caudal regions, expression of AP-2 transcripts was noted in the anterior half of each somite coincident with sites of motor axon outgrowth (Figs. 8A, 8D, and 8E; Keynes and Stern, 1984).

At stage 24 the level of AP-2 transcripts increased in the mesenchyme and ectoderm across the frontonasal mass and lateral nasal prominences (Figs. 5C, 5D, 6C, and 6E). The maxillary prominences AP-2 expression was restricted to two patches, an intense lateral region and a less intense area in the medial and caudal aspect of the maxilla (Figs. 5C, 6C, 6E, and 8A). The expression in the mandibular prominence decreased from stage 20 onwards and by stage 24 expression was limited to the auricular hillock that will give rise to the external ear and a patch of mesenchyme about 500 μm away from the midline on the stomodeal side of the prominence (Figs. 5C, 6C, and 8A and data not shown). In the 2nd branchial arch expression was restricted to the caudal border (Figs. 5C and 8A). There is a gradient of expression of AP-2 in the in two lateral stripes along the hindbrain ranging from very weak signal in r1 and r2 to strong signal in more caudal rhombomeres (Fig. 5C and data not shown). Expression was also noted in the junction of the mesencephalon and diencephalon (data not shown).

At stage 28 transcripts were concentrated along the caudal edge of the frontonasal mass and medial side of the lateral nasal prominences (Figs. 5D and 6E). In the maxillary prominence there was strong expression in the medial and caudal part of the prominence as well as the lateral region that will form the lateral aspects of the upper beak (Fig. 6E). There was minimal expression in the mandibular mesenchyme, except in the anterior midline (Fig. 6E).

AP-2 Is Down-Regulated in the Frontonasal Mass and Lateral Nasal Prominence in Retinoic-Acid Treated Embryos

The mechanism for retinoic acid-induced facial clefts may involve changes in expression of AP-2. In embryos treated with beads soaked in 5 mg/ml of retinoic acid placed in the right nasal pit, expression was significantly down-regulated in the lateral nasal prominence within 8 hr (Compare Fig. 6A with 6B; $N = 3$) and completely down-regulated in the lateral nasal prominence within 16 hr (Fig. 6D; $N = 4$). The morphology of the face was severely affected 48 hr after bead implantation such that the corners of the frontonasal mass were pinched and the right nasal pit was nearly obliterated (Fig. 6F). However, the two expression domains in the maxillary prominences remained unchanged in retinoid-treated embryos (compare Fig. 6E with 6F). The frontonasal mass normally has increased AP-2 expression at stage 24, however in retinoic acid treated embryos this increase does not occur (compare Fig. 6C and D).

Programmed Cell Death in Retinoic Acid-Treated Embryos

We examined retinoic acid-treated and nontreated embryos for cells undergoing programmed cell death. Nontreated embryos exhibited several localized regions of increased program cell death including the junction of the lateral nasal and maxillary prominences, the midline and caudal edge of the mandibular prominence, and the trigeminal ganglia (Figs. 7A–7C and data not shown). The retinoic acid-treated embryos had localized increase in cell death on the side where the bead was placed extending across the caudal edge of the frontonasal mass (Figs. 7A and 7B). Sections of retinoic acid-treated embryos 24 and 48 hr after retinoic acid beads were implanted, revealed a small proportion of mesenchymal cells and a localized region of ectoderm had increased cell death (Fig. 7D and data not shown).

Expression of AP-2 in the Limb Buds Is Associated with Distal Outgrowth

The first stage at which AP-2 expression could be detected in the limb buds was stage 17–18. During early budding stages of limb formation, both the wing and leg buds expressed high levels of AP-2 in the distal mesenchyme (Figs. 8D and 8E, left wing bud, and data not shown). In addition, sections of the wholemount *in situ* embryos revealed that the dorsal and ventral ectoderm express AP-2 but not the AER (data not shown). By stage 24 expression was not restricted to the distal mesen-

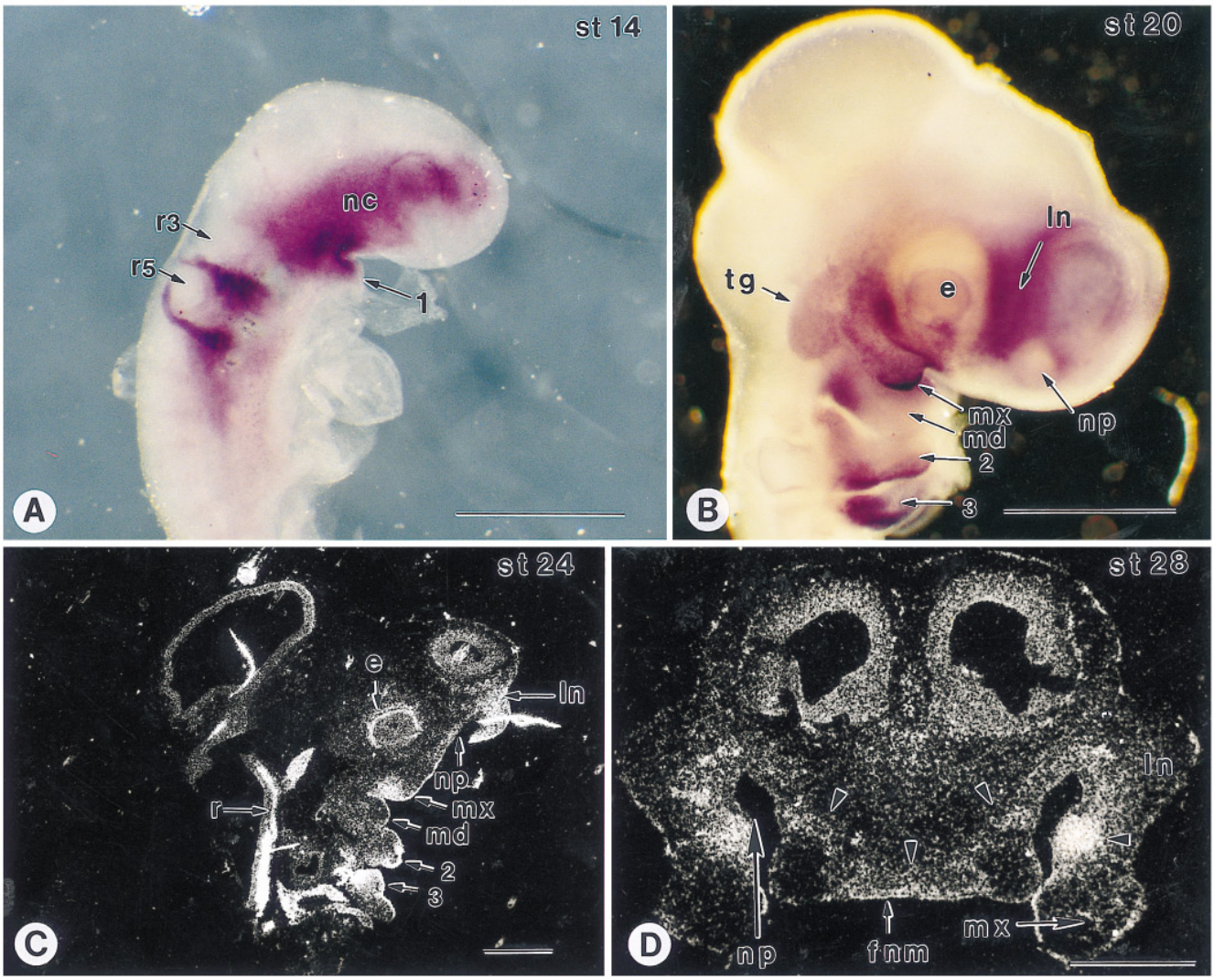


FIG. 5. AP-2 expression in the craniofacial region during face development. All panels photographed with dark-field illumination. C and D used radiolabeled probes on sectioned embryos. Scale bar, 1 mm in A and B; 0.5 mm in C and D. Key: (e) eye, (fnm) frontonasal mass, (ln) lateral nasal prominence, (mx) maxillary prominence, (md) mandibular prominence, (np) nasal pit, (op) otic pit, (tg) trigeminal ganglion, (2, 3) 2nd and 3rd branchial arch. (A) Stage 14. Expression skirts the otic vesicle and a stream of expressing cells is just entering the first branchial arch. There is also expression in the two streams of cells exiting r4 and r6. (B) Stage 20. Expression in the trigeminal ganglion, caudal edge of the maxilla, lateral nasal prominence, and the 2nd and 3rd branchial arches. There is lower expression in the nasal pit and in the mandibular prominence. (C) Stage 24, parasagittal section. Expression is present in the lateral nasal prominence, the caudal edge of the maxilla, second branchial arch, the rhombencephalon, and the surface ectoderm. (D) Stage 28, frontal section. Expression across the frontonasal mass in a chevron shape (arrowheads) and the medial side of the lateral nasal prominences.

chyme but also spread along the anterior margin of the wing and leg buds (Figs. 8A–8C). A patch of cells in the proximal ventral surface expressed high levels of AP-2 at stage 24 and stage 28 leg buds (Fig. 8B and data not shown).

In order to see whether AP-2 is involved in limb outgrowth we stripped the AER of stage 20 limbs and examined gene expression at defined intervals following ridge removal. AER removal is known to truncate limb outgrowth. We observed a rapid, partial, down-regulation of

AP-2 gene expression 4 hr following ridge removal (Fig. 8D); however, 2 hr later there was nearly complete loss of gene expression in limb bud mesenchyme (Fig. 8E). AP-2 transcripts were not present in limb mesenchyme 8 hr or more following ridge removal (Figs. 8F and 8G).

It is known that FGF-4 can replace the AER and stimulate the formation of distal cartilage elements (Niswander *et al.*, 1993). The application of an FGF-4 soaked-bead to the apex of a stripped limb bud encouraged the formation of the radius and ulna in every case, whereas untreated limb buds only form the most proximal part of the humerus (Wilke and Richman, unpublished data). We found that similar to the AER, FGF-4 maintained AP-2 expression in the distal limb mesenchyme (Fig. 8C).

DISCUSSION

The AP-2 Gene Family

There are now three members of the AP-2 gene family, AP-2α, AP-2β, and AP-2γ (Moser *et al.*, 1995; Chazaud *et al.*, 1996; Oulad-Abdelghani *et al.*, 1996; Williamson *et al.*, 1996), and these have been mapped to three separate chromosomes in human and mouse. The expanding gene family and the fact that several splicing variants have been identified in the mouse (Meier *et al.*, 1995) and human (Buettner *et al.*, 1993) offer opportunities for various AP-2 proteins to dimerize *in vivo* (Williams and Tjian, 1991a, 1991b; Bosher *et al.*, 1996). We have cloned AP-2α from the chicken but it is likely that other genes and isoforms of AP-2 exist in the chicken. Our probe would recognize all forms of chicken AP-2α since it spans the highly conserved DNA binding and dimerization domains. In the mouse there are differences in expression between the AP-2α, AP-2β, and AP-2γ/AP-2.2 mRNAs (Moser *et al.*, 1995, 1997; Chazaud *et al.*, 1996). It is therefore likely that chicken AP-2α is expressed in a subset of cells and tissues that we have detected with our *in situ* hybridization probe.

AP-2 Expression and Origin of Surface Ectoderm and Ectodermally Derived Structures

The *in situ* hybridization studies show that there is a correspondence between the horse shoe-shaped part of the epiblast that expresses AP-2 and that which gives rise to the surface ectoderm, as well as the cranial ganglia (Rosenquist, 1966; Hatada and Stern, 1994). AP-2 continues to be expressed in the lateral ectoderm adjacent to neural plate but not in the neural plate itself similar to the mouse (Mitchell *et al.*, 1991; Chazaud *et al.*, 1996). Neural crest cells will form from the lateral edges of the neural folds once the neural tube has formed (Selleck and Bronner-Fraser, 1995) thus the AP-2 expressing region includes presumptive neural crest. However, at these early stages of neurulation, AP-2 is not completely restricted to premigratory neural crest cells.

AP-2 Is a Marker for Migratory Cranial Neural Crest Cells

AP-2 is a very good marker for migratory neural crest and cranial neural crest derivatives. Once neural tube closure has occurred AP-2 expression is closely correlated with sites of cranial neural crest cell migration as detected with HNK-1 antibodies (Babich and Richman, unpublished data) and as mapped with DiI (Lumsden *et al.*, 1991), [^{3}H]-TdR (Noden, 1975), and in quail-chick chimeras (LeLièvre, 1978; Noden, 1978). The crest-free regions adjacent to r3 and r5 are also negative for AP-2 expression. We did note that there is expression of AP-2 in the dorsal midline of r3 and r5, but no AP-2 expressing cells are seen adjacent to the rhombomeres. This correlates well with the *in vitro* observations that r3 and r5 have the potential to give rise to neural crest cells (Graham *et al.*, 1993). There is evidence showing that r3 and r5 neural crest are undergoing increased programmed cell death (Graham *et al.*, 1994, 1993) and that a portion of the neural crest cells originating in r3 and r5 move in a cranial or caudal direction and exit through r2 and r4 (Sechrist *et al.*, 1994). Our data shows that AP-2 is expressed in neural crest derived from r3 and r5; however, AP-2 transcripts are associated with vital cells, not dying cells. In the transgenic AP-2α $-/-$ mice, the absence of AP-2α protein is associated with increased cell death in the neural crest (Schorle *et al.*, 1996). Thus AP-2 may be a marker for the proportion of r3 and r5 neural crest cells that remain vital. It would be necessary to labeling r3 or r5 neural crest cells with DiI and then carry out *in situ* hybridization with the AP-2 probe in order to determine whether this was true.

AP-2 Expression Is High in Structures Derived from Cranial Neural Crest

AP-2 expression continues to be high in facial mesenchyme that will ultimately form the skeletal tissues. The mesenchymal tissue in the frontonasal region is derived from an intermingling of cranial neural crest cells and mesoderm originating in the somitomeres while in the branchial arches the mesodermal and ectomesenchymal cells segregate and the central core is derived from the somitomeres (Trainor and Tam, 1995). It is interesting to note that AP-2 expression is homogenous in frontonasal and lateral nasal prominence mesenchyme but in the branchial arches expression is highest in the periphery corresponding to the neural crest origins of these mesenchymal cells. In addition, the trigeminal ganglion is partly derived from neural crest cells and this ganglion expresses high levels of AP-2 in both chick and mouse (Mitchell *et al.*, 1991; Chazaud *et al.*, 1996).

Down-regulation occurs in the maxillary and mandibular prominences after stage 20. The medial, caudal regions of the maxillary prominences form the palatal shelves, whereas the lateral regions form the quadrojugal and pterygoid bones. AP-2 expression in the maxilla may be necessary for correct patterning of the bones. Decrease in AP-2 expression in the mandibular prominences is more complete. The only region that retains high levels of expression is the auricular hillock which forms the external ear and a curious patch on the stomodeal side of the mandibular prominence. Timely down-regulation of AP-2 may be necessary for patterning the mandibular skeletal elements.

Retinoic Acid-Induced Clefting Is Correlated with Down-Regulation of AP-2

We have created a specific defect in the upper beak by local application of retinoic acid to the nasal pit, long after neural crest cell migration has ceased. The embryos have decreased expression of AP-2 in the frontonasal mass and lateral nasal prominences, both regions known to be targets of retinoic acid (Tamarin *et al.*, 1984; Wedden and Tickle, 1986; Wedden, 1987; Richman and Delgado, 1995; Richman, unpublished data). The maxillary and mandibular prominences are known to be unaffected by retinoic acid as determined by grafting individual prominences from embryos treated with a high dose of retinoic acid (Wedden, 1987; Richman, unpublished results). The maxillary prominences normally fuse with the lateral nasal and frontonasal mass prominences; however, retinoic acid exposure inhibits outgrowth of the frontonasal mass thereby preventing fusion from taking place. There are no changes in AP-2 expression in the maxillary or mandibular prominences following retinoic acid treatment, which is consistent with the lack of response to retinoic acid.

The concentration of retinoic acid that the cells of the face are exposed to is higher than that used to induce duplications of the limb bud but similar to that used to produce truncations of the limb (Tickle *et al.*, 1985). It is unlikely that if we used lower concentrations of retinoic acid that we would see induction of AP-2. Our dose-response studies showed that unilateral facial clefts are induced with lower doses therefore we expect that AP-2 would also be down-regulated in these embryos (Richman and Leon Delgado, 1995).

The Retinoic Acid Defect in the Lateral Nasal and Frontonasal Mass Is Correlated with Localized Increases in Programmed Cell Death

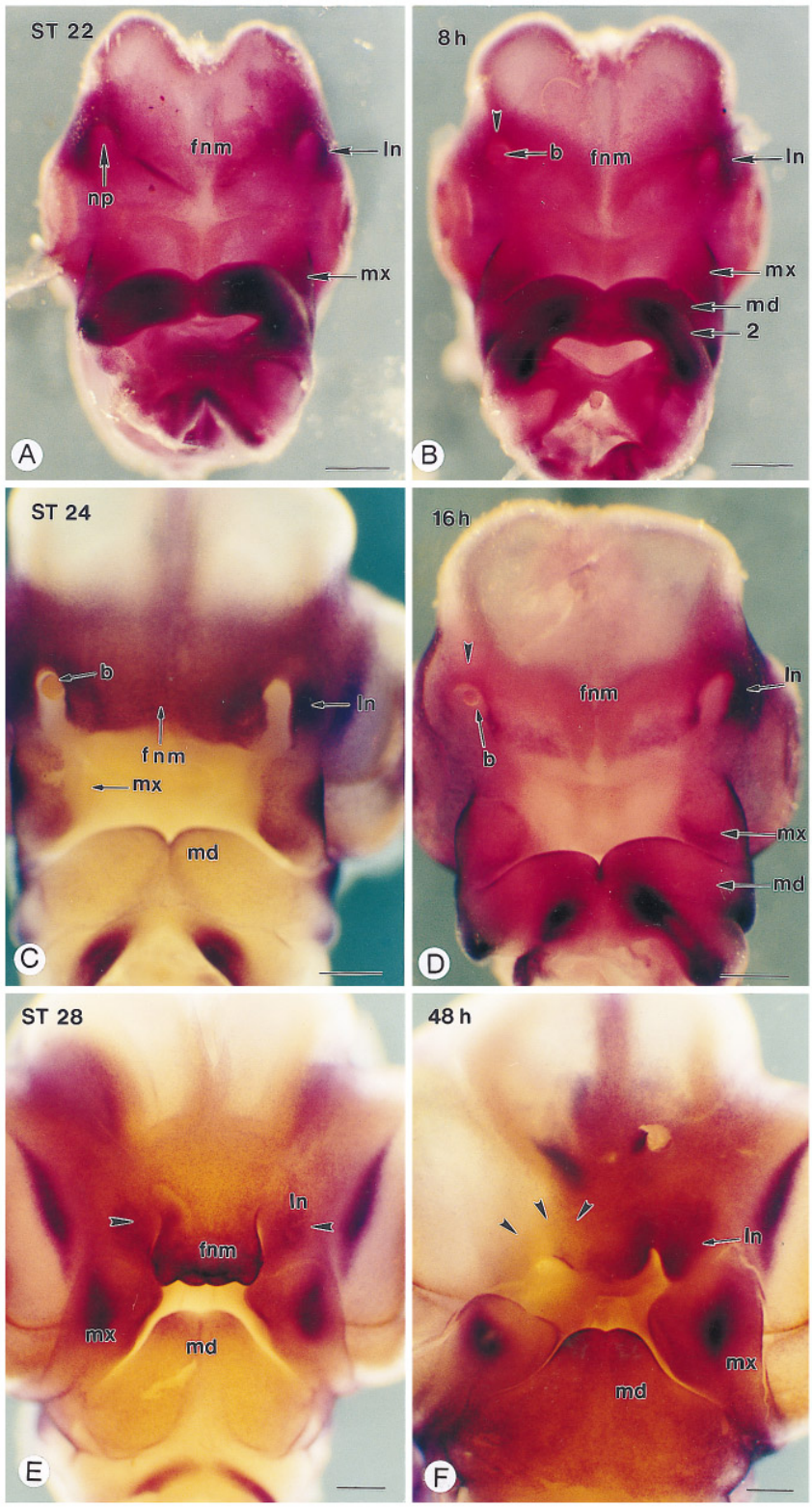
The extent of programmed cell death in the stage 24 chick face has been examined using Nile Blue Sulfate staining (Wedden, 1991; Mina *et al.*, 1995; Barlow and Francis-West, 1997). Several areas of increased staining were noted in both normal and treated embryos and these correspond closely to our TUNEL data. Areas of correspondence include the junction of the lateral nasal and maxillary prominence, the corners of the frontonasal mass, and the caudal edge of the mandibular prominence. Programmed cell death also occurs in the cleft of the mandible, at the caudal edge of the second branchial arch, and in the trigeminal ganglion.

Our study, however, differs from that of Wedden (1991) in that we can detect a localized increase in cell death 24 hr after retinoic acid-bead implantation in both the epithelium and the mesenchyme. Increased numbers of dying cells are observed adjacent to the nasal pit where the retinoic acid bead was implanted. The proportion of dying cells in the mesenchyme is small and is unlikely to account for the significant decrease in AP-2 expression following retinoic acid treatment. In epithelial-mesenchymal recombination studies the target of retinoic acid is known to be the mesenchyme rather than the epithelium (Wedden, 1987). The cell death observed in the ectoderm following retinoic acid treatment is likely to be secondary to changes in the mesenchyme.

Retinoic Acid and AP-2 Transcription

Are the effects of retinoic acid on AP-2 expression in the face regulated via changes in gene transcription? Although

FIG. 6. Retinoic acid down-regulates AP-2 expression. Embryos implanted with beads (arrowhead) soaked in DMSO (C, E), or 5 mg/ml retinoic acid (B, D, F) placed in the right nasal pit at stage 20. All were hybridized with antisense probe for AP-2α. Scale bar, 0.5 mm. Key: (b) bead, (fnm) frontal nasal mass, (ln) lateral nasal prominence, (mx) maxilla prominence, (md) mandibular prominence, (np) nasal pit, (st) stage, (2) 2nd branchial arch. (A) Normal, untreated stage 22 embryo. Expression of AP-2 around the nasal pits. (B) Down-regulation of AP-2 expression in the lateral nasal prominence and lateral edge of the frontonasal mass 8 hr after bead implantation in the right nasal pit (arrowhead). (C) Stage 24 embryo treated with a DMSO-soaked bead. High levels of AP-2 expression are noted in the lateral nasal process and frontonasal mass. Maxillary prominences express AP-2 at the lateral edges and inferior-medial edge. (D) Complete down-regulation of AP-2 16 hr after bead implantation in the right nasal pit (arrowhead). (E) AP-2 expression is seen across the caudal edge of the frontonasal mass 48 hr after a DMSO-soaked bead was implanted in the right nasal pit. There are also AP-2 transcripts in the lateral nasal prominence (arrowheads) and lateral aspect of the maxillary prominences. (F) Forty-eight hours after retinoic acid bead implantation there is significant down-regulation of AP-2 expression in the lateral nasal prominence and right side of the frontonasal mass prominence (arrowheads). There is no change in expression within the maxillary prominences.



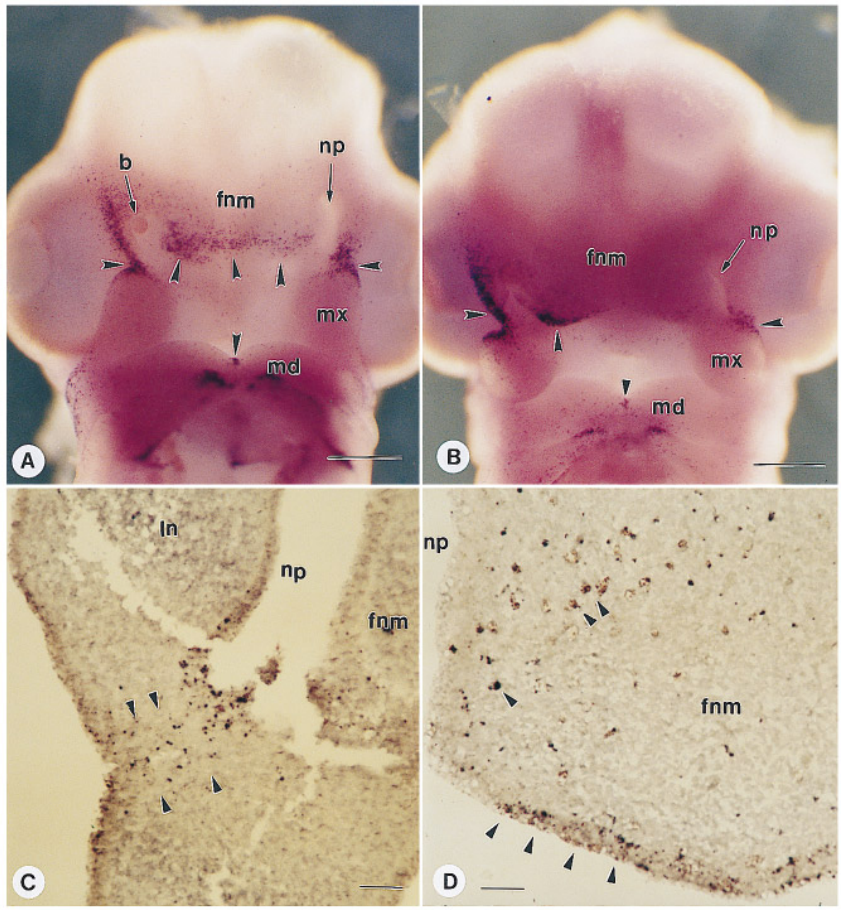


FIG. 7. Cell death in retinoic acid-treated embryos using the TUNEL method. Embryos in A, B, and D were treated with a retinoic acid-soaked bead placed in the right nasal pit at stage 20. A and B were stained as wholemounts, whereas in C and D embryos were sectioned first and then stained. Scale bar, 1 mm in A, B; 250 μm in C, D. Key: (b) bead, (fnm) frontal nasal mass, (ln) lateral nasal prominence, (mx) maxilla prominence, (md) mandibular prominence, (np) nasal pit. (A) Twenty-four hours after retinoic acid bead implantation, punctate staining indicating dying cells is evident around the nasal pit, the junction of the maxillary and lateral nasal prominences, the caudal edge of the frontonasal mass, and the central cleft in the mandibular prominence (arrowheads). (B) A second embryo treated identically to that in A. Similar areas of increased cell death around the right nasal pit are seen as in A (arrowheads). (C) Frontal section. Untreated stage 24 embryo demonstrates a line of dying cells in the mesenchyme between the lateral nasal and the maxillary prominence. (D) Frontal section. Forty-eight hours after retinoic acid bead implantation there is increased cell death in the mesenchyme and epithelium on the right side of the frontonasal mass (arrowheads).

our experiments do not address this issue, there is evidence in other experimental systems that retinoic acid can regulate AP-2 transcription. The expression of AP-2 is up-regulated within 24 hr in teratocarcinomal cells treated with retinoic acid (Williams *et al.*, 1988; Lüscher *et al.*, 1989). A similar increase in transcription was detected in embryonal carcinoma cells of the neuroectodermal lineage but not in cells of the mesoendodermal lineage nor in cultures of primary astrocytes (Philipp *et al.*, 1994). The up-regulation in both cases is at the transcriptional level but the kinetics imply that the AP-2 induction is a secondary response to activation of other genes and may be associated with cell differentiation. AP-γ/2.2 expression in P19 embryonal carcinoma cells is increased 30 min following retinoic acid treatment due to an increase in transcription rate (Oulad-Abdelghani *et al.*, 1996).

In the present study we clearly observed down-regulation of AP-2 following retinoic acid treatment. Changes in AP-2 expression in facial cells treated with retinoic acid precedes any changes in morphology. One explanation for the

difference between our results and those observed in cell culture may be that we are not inducing cell differentiation prior to changes in gene expression. Rather than being involved with cell differentiation *in vivo*, we hypothesize that AP-2 down-regulation affects outgrowth of facial prominences. The 8-hr delay in AP-2 down-regulation observed in retinoic acid-treated faces leaves open the possibility that other genes are activated before we see changes in AP-2 expression.

If AP-2 down-regulation in the face is not the primary effect of exogenous retinoids, what genes might be upstream from AP-2? The sequence of the human (Bauer *et al.*, 1994) and chick AP-2 promoters has been studied (Creaser *et al.*, 1996) and no retinoic acid response elements (RAREs) have been identified. However, there are functionally active AP-2 binding sites indicating that autoregulation can occur (Bauer *et al.*, 1994). We cannot rule out the possibility that AP-2 genes are influenced by another gene upstream in the developmental pathway that contains a RARE. We know that retinoic acid receptors and cellular retinoic acid binding proteins are expressed at high levels in the chick facial prominences (Rowe *et al.*, 1991, 1992; Smith and Eichele, 1991; Vaessen *et al.*, 1990; Maden *et al.*, 1991) thus the door is left open for gene interactions via RAREs.

Msx-1 and Other Potential Downstream Target Genes of the AP-2 Transcription Factor

Msx-1 and Msx-2 are expressed in similar regions to AP-2. In particular there is high expression of all three genes in the lateral nasal prominences and corners of the frontonasal mass (Brown *et al.*, 1996) as well as the distal mesenchyme of the limb bud (Davidson *et al.*, 1991; Yokouchi *et al.*, 1991; Nohno *et al.*, 1992; Mitchell *et al.*, 1989; Chazaud *et al.*, 1996). Pronounced down-regulation of Msx-1 and Msx-2 expression was observed in the lateral nasal prominences following exposure to exogenous retinoic acid at stage 20 (Brown *et al.*, 1996) and similarly, Msx-1 was down-regulated in the limb bud following retinoic acid bead implantation (Yokouchi *et al.*, 1991). In contrast, the frontonasal mass often retained expression of both genes up to 48 hr following bead implantation. Our results show that AP-2 expression was nearly absent from the right side of the frontonasal mass in addition to the lateral nasal prominence 16 hr following bead implantation. Thus AP-2 expression in the frontonasal mass appears to be more responsive to retinoic acid than does Msx-1. Two possible explanations should be considered. The first is that Msx-1 responds directly to retinoic acid via a cis-regulatory element that has been identified in the promoter (Shen *et al.*, 1994). Therefore, the timing of response of the Msx-1 gene in relation to the AP-2 gene in retinoic acid-treated embryos may be coincidental.

The second possibility is that AP-2 regulates *Msx-1*. There is a binding site for AP-2 in the Msx-1 promoter (Kuzuoka *et al.*, 1994) although functional activation has not been tested. The expression of *Msx-1* was studied in the transgenic AP-2α $-/-$ embryos (Schorle *et al.*, 1996). *Msx-1* transcripts were absent in the medionasal region of $-/-$ mice but were still present in the lateral nasal, maxillary, and mandibular prominences. The pattern of expression was different compared to $+/-$ littermates. *Msx-1* was not restricted to the tips of the prominences but was concentrated on the lateral aspects, as though the direction of growth of the facial prominences was changed. This correlated with the midline cleft of the facial bones in older embryos. It is possible that AP-2α was required for establishing the pattern of *Msx-1* transcripts in the facial prominences. The reason that *Msx-1* was not completely down-regulated in the transgenic mice may be that other AP-2 genes also expressed in the face compensated for the lack of AP-2α (AP-γ/2.2; Chazaud *et al.*, 1996).

Lack of Response of the Mandibular Prominences to Retinoic Acid May Be Due to a Deficiency in AP-2 Transcripts

The basis for the lack of response of the mandibular prominences to retinoic acid could be explained by the down-regulation of AP-2 transcripts shortly after stage 20. Even when a retinoic acid bead is placed in direct contact with the mandibular prominence there is no effect on morphogenesis or on Msx-1 expression (Brown *et al.*, 1997). It is possible that embryos younger than stage 20 treated with retinoic acid would have mandibular defects since expression in the first branchial arch is pronounced from stage 14 to stage 20. We have found that embryos treated at younger stages have pronounced defects in Meckel's cartilage and other hypobranchial structures (Saurette and Richman, unpublished results).

AP-2 and Limb Outgrowth

AP-2 is one of several signals in the limb that are associated with outgrowth. The lack of outgrowth in limb buds stripped of their AER is strongly correlated to down-regulation of AP-2 just as truncation of the upper beak by retinoic acid is associated with a down-regulation of AP-2. The time course of down-regulation is very similar to that documented for Cek-8 (chicken embryo kinase-8; Patel *et al.*, 1996). The changes in gene expression precede major changes in the size of the limb bud and the timing of down-regulation is identical to that observed in the retinoic acid-treated face. We have also demonstrated that AP-2 expression in distal limb bud mesenchyme can be maintained by FGFs as are *Msx-1* (Vogel *et al.*, 1995) and Cek-8 (Patel *et al.*, 1996). Other genes expressed in the progress zone of the limb (Summerbell *et al.*, 1973) may be involved in the regulation of AP-2 expression and

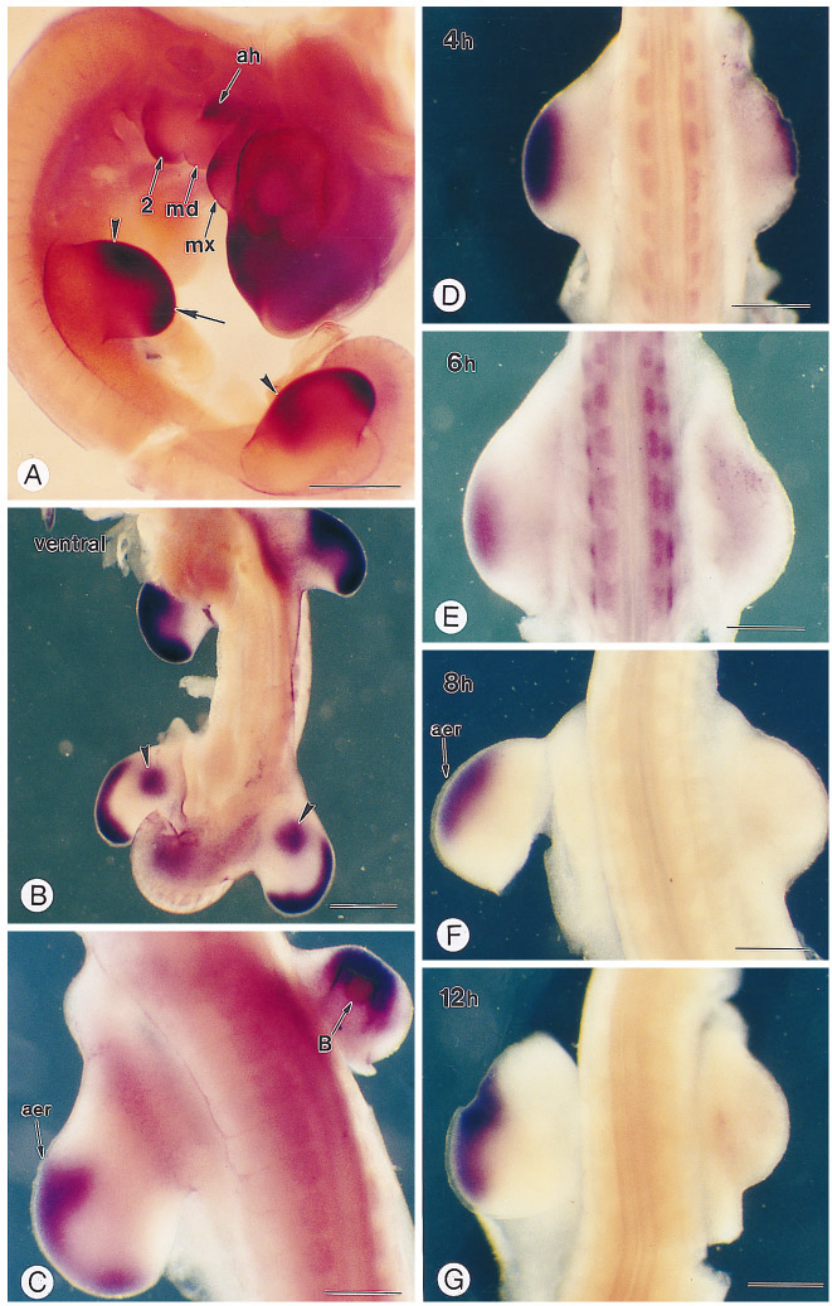


FIG. 8. AP-2 expression in limb buds is associated with distal outgrowth. All embryos hybridized to an antisense probe for chick AP-2α. A is a sagittal view, B is a ventral view, and C–F are dorsal views of the embryo. Scale bar equals 1 mm for A, B; 0.5 mm for C–G. Key: (aer) apical ectoderm ridge, (ah) auricular hillock, (b) bead, (md) mandibular prominence, (mx) maxillary prominence, (2) second branchial arch. (A) Stage 24. Expression in the distal (arrow with tail) and anterior mesenchyme of limb buds (arrowheads), the caudal edge of the second branchial arch, the auricular hillock, the caudal edge of the maxillary prominence, and the dorsal root ganglia. (B) Stage 24. Additional expression domains are seen on the ventral side of the leg buds. (C) Stage 24. Right limb has had the AER removed at stage 20, a bead soaked in FGF-4 was stapled to the apex of the wing bud and the embryo was incubated for 24 hr. There are high levels of AP-2 transcripts surrounding the bead. The left wing bud has not been manipulated and the AER is devoid of AP-2 transcripts. (D) Stage 21. Four hours since AER removal, there is partial down-regulation of AP-2 in the mesenchyme. (E) Stage 21. Six hours since AER removal, AP-2 expression is slightly above background levels in the right limb bud. (F) Stage 22. Eight hours since ridge removal, there is complete down-regulation of AP-2 in the right limb bud. (G) Stage 22.5. Twelve hours since ridge removal, AP-2 transcripts are completely absent in the right limb bud. The right limb bud is noticeably smaller than the left limb bud. The left limb bud was injured during *in situ* hybridization.

these molecular relationships will need to be investigated in more detail.

The effects of disrupting AP-2α in transgenic mice on limb development were relatively mild. Null-mutant embryos displayed forelimb phocomelia (the radius was absent in 18 of 28 −/− fetuses; Zhang *et al.*, 1996). On a different genetic background the AP-2 −/− mice have general retardation of the development as well as loss of the radius (P. J. Mitchell, personal communication). The phocomelia implies that there was a temporary decrease in the number of cells being produced by the highly proliferative progress zone (Wolpert *et al.*, 1979). The subsequent recovery of distal limb outgrowth could have been orchestrated by the other forms of AP-2 that are expressed in the progress zone but were not disrupted in the AP-2α −/− embryo (AP-2.2/γ; Chazaud *et al.*, 1996).

In addition to outgrowth of limb buds, AP-2 may play a role in patterning the digits. At stage 24 there is a distinct zone of expression extending from the progress zone along the anterior margin. This pattern of expression is not similar to other genes previously described to be expressed along the anterior margin of the limb bud (BMP-4, Francis-West *et al.*, 1994; Francis *et al.*, 1994; Msx-1, Ros *et al.*, 1992; Dlx-5, Ferrari *et al.*, 1995). In addition, hind limbs have a prominent patch of expression in the ventral surface just as seen in mouse embryos (Mitchell *et al.*, 1991; Chazaud *et al.*, 1996). These domains of expression may indicate a role for AP-2 in dorso-ventral patterning of proximal structures in addition to Wnt-7a and Lmx-1 (Parr and McMahon, 1995; Riddle *et al.*, 1995; Vogel *et al.*, 1995).

Function of AP-2 in Mammalian Craniofacial Development

The function of AP-2 has been studied in knockout mice with a disrupted AP-2α gene. The facial phenotype is first evident when the primary palate is forming (E 9.5) and is characterized by loss of the medial nasal prominences and slightly smaller mandibular prominences. Once ossification has occurred, the maxillary and mandibular bones appear to fuse to each other and there is a prominent midline cleft. The trigeminal ganglion is also greatly reduced in size in the −/− mice. One possible explanation for the defects is that AP-2 is required for neural crest cell specification and normal migration. Our results highlight the expression of AP-2 in migrating cranial neural crest cells and support the hypothesis that neural crest cells are affected in the −/− mice. Zhang *et al.* (1996) used an antibody to NCAM to identify migratory neural crest cells and found that the pattern of expression seemed to be altered in −/− embryos, but there was no obvious deficiency of neural crest cells. In contrast, the cell death studies of Schorle *et al.* (1996) illustrate that there is increased programmed cell death at the dorsal edges of the neural folds in −/− embryos. Thus it is possible that there is a selective loss of neural crest cells that contribute to the facial mesenchyme, a loss that may not be detected with antibody staining. Finally, it is possible that the specification of neural crest cells (as in Noden, 1983) be could be disrupted in AP-2 −/− mice, a hypothesis that remains to be tested.

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